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Human Cytomegalovirus and Acute Rejection After Heart Transplantation Are Not Directly Associated

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Retrospective and prospective analyses of heart transplant recipients showed no significant association between acute rejection and the detection of cytomegalovirus (CMV) infection by culture or the polymerase chain reaction (PCR) for viral DNA, neither on grounds of the incidence of both conditions nor in relation to which was diagnosed first in the patient. Semiquantitative PCR of serial blood and endomyocardial biopsy specimens from individual patients revealed different patterns in the development of the viral DNA in the blood and the heart, also clear episodes of CMV infection in CMV antibody-negative recipients of hearts from CMV antibody-negative donors, none of whom went on to develop a CMV-specific antibody response. None of these findings was associated with the development of rejection in the patient. On the other hand, in those patients who did experience rejection, peak levels of CMV DNA in the blood and the heart were usually not reached until 6 weeks or more after transplantation, whereas in those in whom rejection was not detected at all during the period of observation, peak levels of CMV DNA were detected earlier, mainly within the first 6 weeks after transplantation. In several cases, the delayed increase in CMV DNA in those with rejection, albeit not the delay itself, was linked to treatment with steroids. These findings support the view that CMV infection and rejection are independent events, but that the timing of the infection, and whether or not rejection is detected, are indicative of the general status of the immune response in individual patients.

KEY WORDS: human cytomegalovirus, heart transplantation, rejection, endomyocardial biopsy, polymerase chain reaction

INTRODUCTION

Human cytomegalovirus (CMV) infection in non-pregnant immunocompetent individuals is mainly benign, only occasionally giving rise to an infectious mononucleosis-like syndrome. Infection is followed by virus persistence, with episodes of reactivation and recurrence, as with other herpesviruses. Recovery from primary infection is accompanied by a detectable specific antibody response which persists, probably for life, due to continual boosting by reactivation or exogenous reinfection. Use of the polymerase chain reaction (PCR) technique has furnished evidence of CMV DNA in the blood of individuals who are CMV antibody-negative [Bevan et al., 1991; Ehrnst et al., 1995; Stanier et al., 1989; Taylor-Wiedeman et al., 1991; Zhang et al., 1995] but this is controversial [Bitsch et al., 1992; Gerna et al., 1991; Jiwa et al., 1989; Urushibara et al., 1995].

CMV infection in immunocompromised patients, including transplant recipients, can give rise to a range of complications which, in the case of interstitial pneumonitis, may be life-threatening, hence many transplant centres undertake routine virological surveillance by various procedures such as serology, virus culture especially from blood, the antigenemia test, and the detection of CMV DNA or mRNA in blood using the PCR. In heart transplantation, another complication is rejection which, over a period of time, may lead to allograft vascular disease and death [Oaks and Wallwork, 1993]; patients therefore undergo continual monitoring by histopathological examination of endomyocardial biopsy specimens [Billingham et al., 1991]. Two rejection syndromes are recognized, namely, acute rejection within the first 3 months or so after transplantation, and chronic rejection which has a more long-term and insidious development. Both have been linked to CMV infection on the basis of detecting viral nucleotide sequences or antigens in situ in the heart [Hendrix et al., 1990; Jäkel et al., 1992; Lowry et al., 1994], or by virtue of showing that patients with rejection are more likely to have undergone recent CMV infection [Grattan et al., 1989; McDonald et al., 1989; Petrie et al., 1987]. This

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association is controversial [Gulizia et al., 1995; Sharples et al., 1991; Skowronski et al., 1993; Stovin et al., 1989] and is made more so by the fact that the standard immunosuppressive treatment for rejection, namely with steroids, increases the likelihood of CMV reactivation and allows increased viral replication.

We investigated previously the relationship between CMV and acute rejection in our heart transplant patients, in a retrospective study on stored endomyocardial biopsy samples, using a nested semiquantitative PCR for CMV DNA, and relating the results to those of virus culture and serology [Fernando et al., 1994]. Significantly more biopsies were positive for both CMV DNA and moderate-severe rejection than for CMV DNA and mild or no rejection; however, there was no significant association between rejection and the culture of virus from urine throat or blood, or between rejection and the detection of virus-specific IgM antibodies. When the results were assessed in relation to donor:recipient (D:R) CMV antibody status, the only significant finding was that positivity for CMV DNA was associated with moderate-severe rejection but only in those patients who themselves were CMV IgG-positive, and their donors, at the time of transplantation (D+R+ patients). A feature of this analysis was that moderate-severe rejection was detected in over half the patients who were serologically D−R− at the time of transplantation; also, CMV DNA was detected in a proportion of the biopsies from the same D−R− patients, despite great care having been taken to exclude contamination in the PCR. A concern was that CMV DNA in biopsy samples simply reflected CMV DNA in the blood perfusing the heart and was not directly indicative of CMV-related events taking place within the heart.

To readdress the relationship between CMV and acute rejection in heart transplantation we have now undertaken a prospective study to compare levels of CMV DNA concurrently in both endomyocardial biopsy specimens and blood. This has provided evidence that levels of CMV DNA in the heart do not necessarily reflect those in the circulation, CMV infection is not uncommon in D−R− patients, rejection and CMV infection are not causally linked but CMV infection tends to develop later in those who experience rejection.

MATERIALS AND METHODS

Tissue Samples

Heparinized blood (3–5 ml) was centrifuged at 2,000 rpm for 3 min (MSE Centaur centrifuge, Fisons, Loughborough, UK) and the visible buffy coat layer was collected into 2 ml of lysis buffer [Fernando et al., 1994]; the liberated nuclei were washed in fresh lysis buffer, with low-speed centrifugation, until no hemoglobin was seen, then resuspended in extraction buffer containing proteinase K [Fernando et al., 1994] and heated at 65°C for 2 hr followed by boiling for 10 min.

Percutaneous transvenous right ventricular endomyocardial biopsies were collected weekly for the first 6 weeks post-transplantation and every other week up to 12 weeks except when contraindicated on clinical grounds. The samples (1.1–8.5 mg, mean 4.4 mg) were placed immediately onto gauze moistened with sterile saline and processed, within an hour, for extraction of nucleic acids, as described [Fernando et al., 1994].

Donor mediastinal lymph nodes were snap-frozen in liquid nitrogen as soon as possible after collection. To extract DNA, 0.1–0.3 g of tissue, devoid of fat, was ground in 0.5 ml of Trysol (Life Technologies, Paisley, Scotland) in a 1.5 ml tube using a fitted disposable pestle; 0.5 ml of fresh Trysol and 0.2 ml of chloroform were added and then the DNA was extracted according to the manufacturer's instructions.

The concentration of the extracted DNA, measured by fluorimetry, was adjusted to 100 ng/μl, wherever possible, in distilled water in the case of DNA from blood; the biopsy samples seldom yielded greater than 100 ng/μl.

Semiquantitative CMV DNA PCR

The nested PCR, amplifying a 168 bp DNA product from the *mtrII* region of the CMV genome, has been described [Fernando et al., 1994]. The first-round amplification included, besides the CMV-specific primers, primers for a 360 bp region of the human histidyl-tRNA synthetase (HRS) "housekeeping" gene [Taylor-Wiedeman et al., 1991]. The sensitivities of the nested *mtrII* gene PCR and the single-round HRS gene PCR were 1 and 219 copies, respectively, by limiting-dilution titration of the relevant target sequence cloned into the TA plasmid vector (Invitrogen, Leek, The Netherlands).

Samples were tested also by nested PCR for the CMV major immediate-early (MIE) gene (216 bp product spanning nucleotides 2607–2823 [Akrigg et al., 1985]; primer sequences of Vinogradskaya et al. [1995]) and the CMV glycoprotein H (gH) late gene (256 bp product spanning nucleotides 206–452 [Pachl et al., 1989]; primer sequences of Chou [1992]).

For testing by nested PCR, each sample that contained in excess of 100 ng/μl total DNA was diluted to this value, in distilled water, and the concentration remeasured; samples containing less than 100 ng/μl were tested undiluted. Each sample was also initially screened at a dilution of 1:10, in order to guard against false-negative results due to inhibitors. Samples which gave positive reactions for both the HRS gene and the CMV gene were then titrated by the nested PCR for the CMV gene, using serial 10-fold dilutions, in duplicate; the highest dilution which gave a positive result in one or both duplicates was considered to be the end-point. From this was calculated the number of copies of viral DNA in 100 ng of the total sample DNA.

Each CMV *mtrII* PCR product DNA was confirmed as such by nucleotide sequencing [Boriskin et al., 1994]. Some sequences contained a single base change of A to G, at position 413 [Razzaque et al., 1988], which created a unique restriction site for the *BspEI* endonuclease which allowed the 168 bp product DNA to be cleaved into two subfragments of 93 and 75 bp (Fig. 1). Digestion

Fig. 1. The demonstration of two CMV variants by digestion of the *mtrII* PCR product DNA with the restriction enzyme *BspEI*. In the case of the G variant the enzyme produces two fragments of 93 and 75 bp (**lane 3:** undigested PCR product DNA, 168 bp; **lane 4:** digested), whereas for the A variant the cleavage site is missing (**lane 1:** undigested; **lane 2:** digested). Lanes 5–8 show a sample containing both variants (**lane 5:** undigested; **lanes 6–8:** digested neat and in 5-fold serial dilutions). **Lane M:** DNA molecular size markers.

revealed whether there was one variant alone, or both together, in the original samples.

Data Analysis

CMV DNA levels were plotted cumulatively as $\log_{10}$ number of copies of *mtrII* target DNA vs. weeks after transplantation using the application "Cricket Graph" for the Macintosh. Quantitative comparisons of cumulative plots were made by cutting out and weighing the area under the curve, from a standard printout, in each case. Evaluation of frequency data for significance was by the chi-square test.

Virological Surveillance and Clinical Management

Details are described by Fernando et al. [1994] and by Steel and Murday [1996]. Procedures for CMV culture by cytopathology after 3 weeks incubation and by immunocytochemical staining after 48 hr incubation are given by Steel et al. [1988]. Serological monitoring for CMV infection was by in-house and commercial enzyme immunoassays.

Specific anti-rejection treatment was instituted if the biopsy was graded 3a or worse. In general, cyclosporin and azathioprine were maintained at standard doses with or without additional methylprednisolone, equine anti-lymphocyte globulin, or rabbit anti-thymocyte globulin.

RESULTS

CMV Infection and Rejection: Retrospective Analysis

The clinical records of 90 transplant patients were analyzed in relation to 1) the D:R status (positive or negative) for CMV IgG antibody at the time of transplantation, 2) whether CMV was cultured from the patient's blood, and 3) whether there was evidence of severe rejection in the first 12 weeks after transplantation. Fifty (56%) patients became culture-positive, of whom 27 (30% overall) developed rejection and 23 (26% overall) did not; of the 40 who remained culture-negative, 26 (29% overall) developed rejection (Table I); there was no significant association between CMV infection and rejection ($P > 0.05$). Sixty-six (73%) of the patients were initially either D+R+, D+R− or D−R+ by serology, 49 (54% overall) of whom were positive by culture; of the 24 patients in the D−R− group, only 1 was culture-positive. The number of D+R− patients was small, due to our policy of avoiding this combination where possible, so that the data were combined with those for the D+R+ patients for analysis.

In the 27 patients who became positive for both viremia and rejection, the rejection was detected earlier than infection in 18, and infection first in 9, showing no consistent temporal association between infection and rejection.

CMV Infection and Rejection: Prospective Study

Twenty-seven patients, different from those in Table I, were followed up for 10–12 weeks post-transplantation, with regular testing of anti-coagulated blood by both culture and semiquantitative PCR; endomyocardial biopsy specimens, collected at the same time as the bloods, were also tested by the PCR. None of the patients was in the category D+R−.

Fifteen (56%) of the 27 patients developed CMV by culture, of whom 6 (22% overall) became positive for rejection; 12 patients (44%) remained culture-negative, of whom 6 (22% overall) developed rejection (Table IIa). These findings, like those in the retrospective study, demonstrated no significant association between CMV viremia and rejection ($P > 0.05$). All the culture-positive patients were serologically D+R+ or D−R+ at the time of transplantation; 8 of the 12 who remained culture-negative were in the D−R− group.

By the PCR, 24 (89%) of the 27 patients became positive for CMV DNA in both blood and heart including 9 who were consistently negative by culture; 11 of these 24 (41% overall) developed rejection, the 13 others (48% overall) did not, indicating no significant association between rejection and CMV infection as diagnosed by this more sensitive test ($P > 0.05$; Table IIb); 3 patients remained negative by the PCR and 1 of them developed rejection.

There were 8 patients in the D−R− group, all of them negative by virus culture; 5 developed peaks of CMV DNA in both blood and heart during the period of study. All 8 have remained negative for CMV IgG for more than 5–10 months thereafter.

Analysis of the Aggregated PCR Results for Blood and Biopsy Specimens

In all, 111 blood samples were examined from the 12 patients who developed rejection and 150 were examined

TABLE I. Incidence (%) of CMV Infection, Diagnosed by Culture, and of Rejection, in 90 Heart Transplant Patients According to D:R Status for Antibody to CMV Pretransplantation

Status of patients	CMV infection		No CMV infection		Total
	Rejection	No rejection	Rejection	No rejection	
D+R+ }	17	12	8	1	38
D+R− }	(19)	(13)	(9)	(1)	(42)
D−R+	10	10	4	4	28
	(11)	(12)	(4)	(4)	(31)
D−R−	0	1	14	9	24
		(1)	(16)	(10)	(27)
Total	27	23	26	14	90
	(30)	(26)	(29)	(15)	(100)

TABLE IIa. Incidence (%) of CMV Infection, Diagnosed by Culture, and of Rejection, in 27 Heart Transplant Patients According to D:R Status for Antibody to CMV Pretransplantation

Status of patients	CMV infection		No CMV infection		Total
	Rejection	No rejection	Rejection	No rejection	
D+R+	3	4	1	1	9
	(11)	(15)	(4)	(4)	(34)
D−R+	3	5	0	2	10
	(11)	(18)		(7)	(36)
D−R−	0	0	5	3	8
			(18)	(11)	(29)
Total	6	9	6	6	27
	(22)	(33)	(22)	(22)	(100)

TABLE IIb. Incidence (%) of CMV Infection, Diagnosed by the PCR for CMV DNA, and of Rejection, in 27 Heart Transplant Patients According to D:R Status for Antibody to CMV Pretransplantation

Status of patients	CMV DNA		No CMV DNA		Total
	Rejection	No rejection	Rejection	No rejection	
D+R+	4	5	0	0	9
	(15)	(18)			(33)
D−R+	3	7	0	0	10
	(11)	(26)			(37)
D−R−	4	1	1	2	8
	(15)	(4)	(4)	(7)	(30)
Total	11	13	1	2	27
	(41)	(48)	(4)	(7)	(100)

from the 15 who did not; the proportions positive for CMV DNA in both cases were not significantly different (36% and 40%, respectively; $P > 0.05$; Table III). Likewise, there was no difference in the prevalence of CMV DNA in the endomyocardial biopsy samples from the rejectors and from the non-rejectors (100 and 135 samples, respectively), with 28% and 27%, respectively, being found to be positive ($P > 0.05$; Table III). On further subdividing the data according to the patients' D:R status by serology before transplantation, the only significant difference was in the small number of specimens, blood or heart, from the D−R− patients without rejection, which were positive for CMV DNA ($P < 0.005$; Table III).

Longitudinal Analysis of the PCR Results in Individual Patients

Distinct peaks of CMV DNA were detected in both the blood and the heart in 24 (89%) of the 27 patients. In both of these sites the peaks reached maximum levels of greater than 2 $\log_{10}$ copies per 100 ng of total DNA. Three different patterns were noted in the order in which the peaks appeared, namely, first in the heart (8 patients), first in the blood (5 patients), and both to-

TABLE III. Prevalence of CMV DNA in Heart and Blood Specimens of Patients With and Without Rejection and According to the D:R Status for Antibody to CMV Pretransplantation

D:R status	Patients with rejection		Patients without rejection	
	Heart	Blood	Heart	Blood
D+R+	8/37[a] (22%)	13/41 (32%)	18/45 (40%)	26/50 (52%)
D−R+	10/27 (37%)	15/30 (50%)	19/63 (30%)	32/70 (46%)
D−R−	10/36 (28%)	12/40 (30%)	0/27 (0%)	2/30 (7%)
Total	28/100 (28%)	40/111 (36%)	37/135 (27%)	60/150 (40%)

[a]No. of specimens positive/No. tested.

gether (11 patients): examples are shown in Figure 2 for a selection of D+R+ and D−R+ patients. When a peak developed first in one site, often this had declined to baseline by the time the peak was reached in the second site (Fig. 2b,d–f). The CMV nucleotide sequences detected in the heart and blood of individual patients were always the same.

No one pattern or combination of patterns was associated with the development of rejection nor with the quantity of total DNA in the samples under test; in the latter case, testing by PCR for the HRS "housekeeping" gene indicated that their content of host cell DNA varied by less than 5-fold (see Fig. 4a). Background levels of latent CMV sequences were not a complicating factor in the results because, in the blood and biopsy samples of 10 CMV antibody-positive recipients, the viral DNA was not regularly detected in the first week after transplantation (results not shown). The conclusion was that levels of CMV DNA varied independently in heart and blood thereby representing separate virological events in both these sites.

CMV DNA in Relation to Pretransplantation D:R Status by Serology

Nine (33%) of the 27 patients who were studied prospectively were D+R+ before transplantation, 10 (37%) were D−R+, and 8 (30%) were D−R−; no patients were D+R−. The levels of CMV DNA in heart and blood, for each of these groups, are presented cumulatively in Figure 3; in the D+R+ and D−R+ groups they were between 1.5-fold and 3-fold higher in the blood than the heart. There was evidence of a bimodal distribution of the CMV DNA levels, with one peak occurring at 2–3 weeks after transplantation and the second at 5–8 weeks. Two such peaks were also detected when the data were replotted according to whether the patients were CMV culture-positive (15 patients positive) or had been treated with ganciclovir (6 patients treated) (data not shown in both cases).

CMV DNA in D−R− Patients

CMV DNA was detected in heart and blood in 5 of the 8 (62%) D−R− patients who were studied prospectively and although the cumulative levels in both of these specimens were considerably lower than in the D+R+ and D−R+ groups, there was still evidence of a bimodal distribution (Fig. 3).

In the three examples in Figure 4, CMV DNA was detected as discrete peaks in both heart and blood at later than 5 weeks after transplantation. All three patients developed rejection. The patient in Figure 4a was given 1 unit of screened blood at 1 week after transplantation and 3 units at 5 weeks; a single dose of methylprednisolone was given during week 6, because of rejection, and this was followed by the development of a significant peak of CMV DNA in the heart during the following week and in the blood 3 weeks later. The patient in Figure 4b was also given methylprednisolone after the first diagnosis of rejection, at week 6, and this was followed immediately by a peak of CMV DNA. The patient in Figure 4c developed rejection after the appearance of peaks of CMV DNA in blood and heart at 5–6 weeks. The two other D−R− patients who developed CMV DNA, not shown in Figure 4, gave low peak levels close to 1 $\log_{10}$ copies of total DNA, within the first 4 weeks after transplantation; 1 of them developed rejection, at week 5. One further D−R− patient was negative consistently for CMV DNA but developed rejection at week 6 which merited treatment with methylprednisolone.

The specificity of the PCR results was confirmed in all cases by reamplification of the original DNA, using the same *mtrII* region primers as before, followed by sequencing of the products. Amplification by nested PCR was also carried out with primers specific for the CMV MIE and gH genes. Positive results for all three genes were obtained on testing DNA from the endomyocardial biopsies in three of the patients whose samples contained the highest concentrations of total DNA. This is consistent with the sensitivity of detection for the MIE and gH PCR being less than for the *mtrII* PCR (unpublished observations).

All the D−R− patients had been given CMV antibody-negative screened blood during surgery, but to further examine the likelihood of the PCR procedure detecting passively acquired latent CMV DNA, blood specimens from 5 CMV antibody-positive healthy adults and 12 antibody-positive children were tested, with negative results in all but 1 child aged 3 years.

To assess the reliability of the serology results, the blood samples from the D−R− patients were retested for CMV IgG by both a commercial and an in-house enzyme immunoassay procedure. The patients in Figure 4a and Figure 4b both gave equivocal readings in the second and third blood samples that were collected but thereafter all three have remained negative for CMV IgG for more than 5–10 months of observation.

The pretransplantation blood specimens from the donors for all five D−R− patients who developed peaks of CMV DNA were negative for both CMV IgG and CMV DNA. However, mediastinal lymph node tissue was available from one of these donors and was found positive for CMV DNA by PCR. Restriction analysis of the PCR product DNA from this positive sample showed a

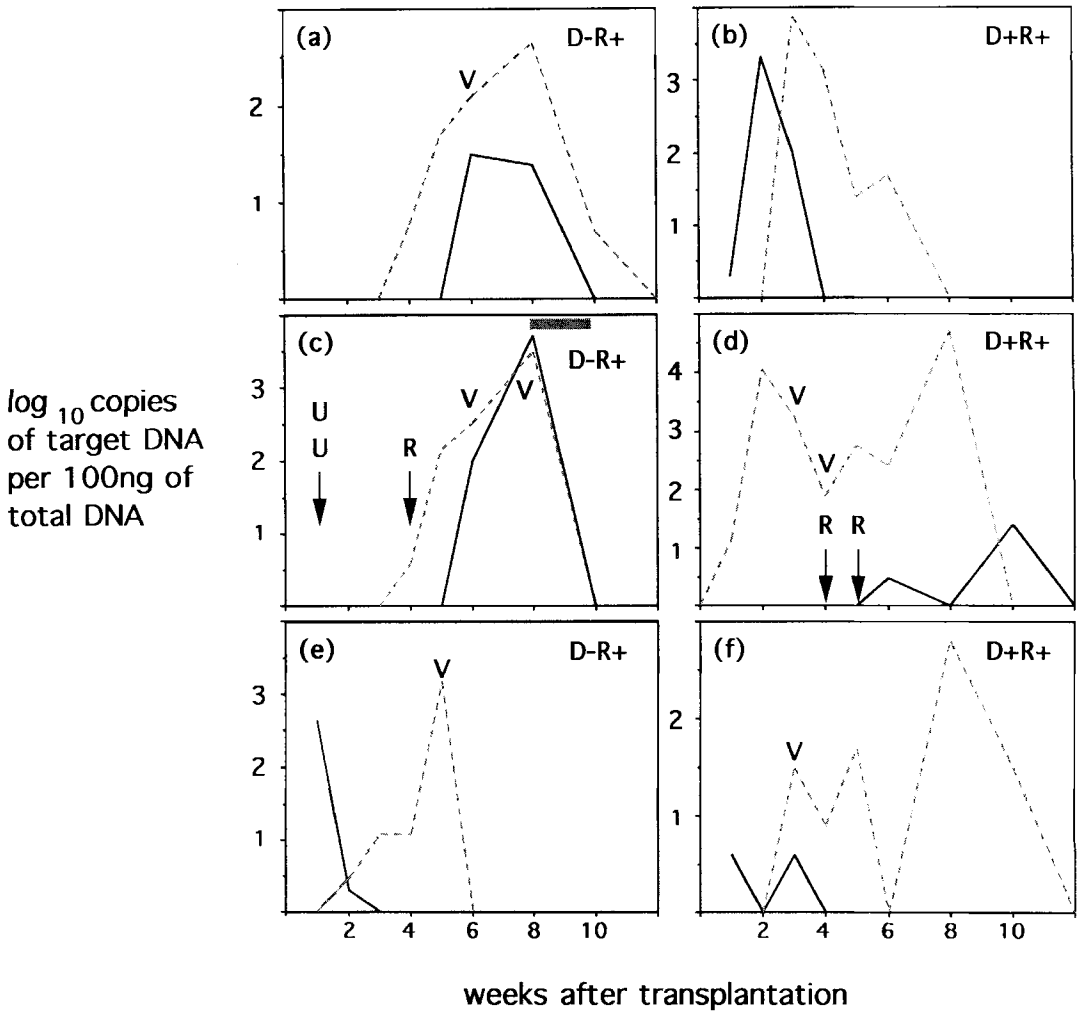

Fig. 2. Development of CMV DNA levels in the heart (solid line) and blood (broken line) of individual patients (**a–f**) with time after transplantation. V = virus isolated in culture; R = rejection; U = unit of blood given; D+ = CMV antibody-positive donor; D− = CMV antibody-negative donor; R+ = CMV antibody-positive recipient; horizontal bar in c indicates treatment with ganciclovir.

mixture of the A and G variants as was detected in the recipient's heart and blood after transplantation (Fig. 4a).

Molecular Epidemiology

Among the 24 CMV DNA-positive patients, 18 demonstrated consistently only the A variant or the G variant (12 and 3 patients, respectively), or both together (3 patients). In two others, the variant after transplantation was different from that in the recipient's blood before transplantation but the same as in the donor's blood. In the remaining four patients, the identity of the variant changed during the post-transplantation follow-up: in two patients from A to G, in one patient from G to A, and in one patient from A+G to A alone; in two of these patients the variant that finally emerged was the same as that in the donor's blood or lymph node tissue pre-transplantation. Thus, infection with more than one strain of CMV was not uncommon; moreover, there was a tendency for the variant in the donor to infect or superinfect the recipient, in some cases eventually supplanting the variant that was present in the recipient from before transplantation.

CMV DNA Levels and Rejection

Levels of CMV DNA were compared cumulatively, in heart and blood, for patients who did or did not develop rejection (Fig. 5). The overall amounts of CMV DNA in both groups of patients were similar. In the patients who did not develop rejection, CMV DNA tended to accumulate within the first 6 weeks after transplantation, such that the cumulative plots for both heart and blood were clearly skewed to the left. However, in those who did develop rejection, CMV DNA tended to accumulate later, at 6–10 weeks after transplantation, in which case the cumulative plots were skewed markedly to the right.

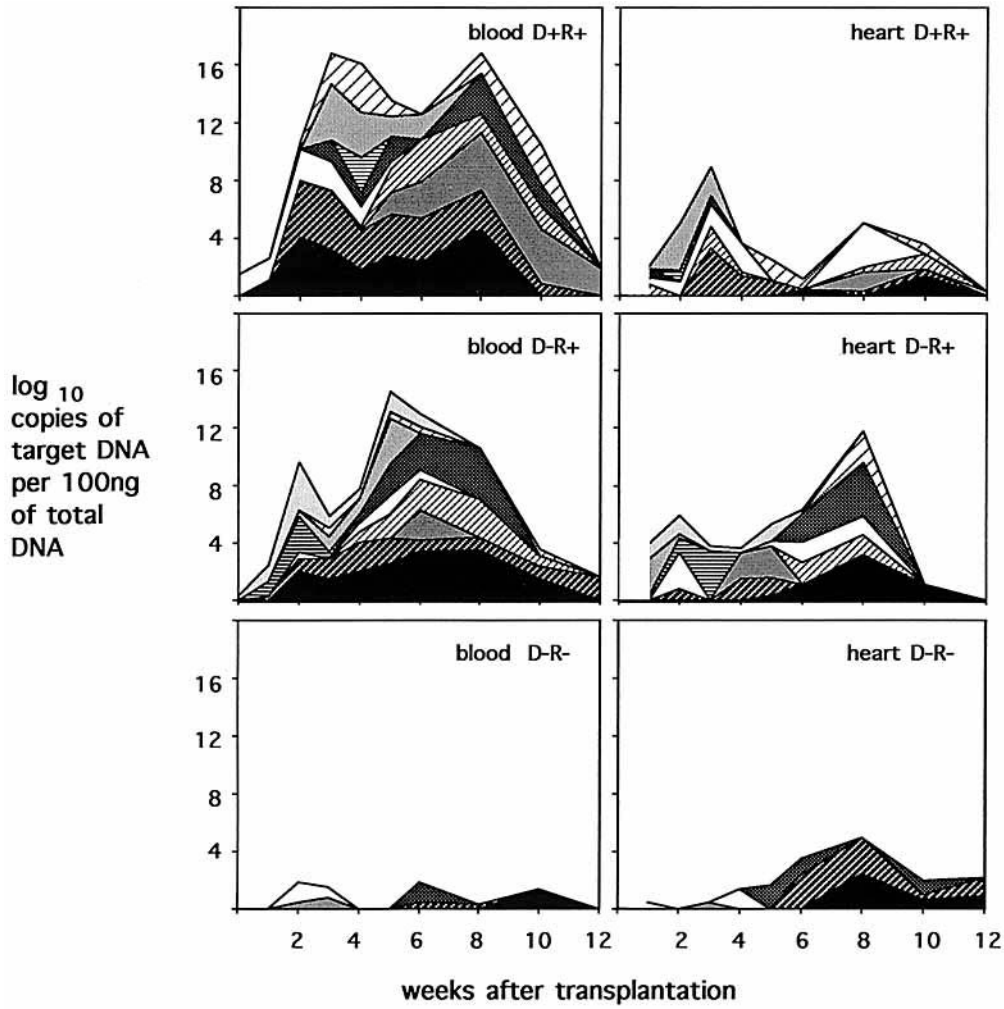

Fig. 3. Levels of CMV DNA in the heart and blood of transplant patients, plotted cumulatively, with time, according to the CMV antibody status of donor (D+ or D−) and recipient (R+ and R−) at the time of transplantation. D+R+: 9 patients; D−R+: 10 patients; D−R−: 8 patients.

This temporal difference between the appearance of CMV DNA in the rejectors and the non-rejectors was particularly evident in the results of the PCR carried out on the hearts. Thus, the early development of CMV infection after transplantation was not associated with rejection, whereas the late development of CMV infection was associated with rejection.

Of the 12 patients who did develop rejection, in 8 it was of grade 3, warranting treatment with methylprednisolone, and in 5 of these the treatment was followed by an increase in CMV DNA. In the 3 others who were treated with methylprednisolone, CMV DNA levels had already become manifest in the 2–4 weeks before the detection of rejection. Thus, although treatment with methylprednisolone did not explain the detection of low levels of CMV DNA within the first 6 weeks or so after transplantation in those who did develop rejection, it did account partly for the high levels of CMV DNA that developed after 6 weeks in the group of patients.

DISCUSSION

Retrospective analysis of data on 90 patients showed that these patients were no more likely to develop rejection in association with CMV viremia (as detected by culture) than either rejection alone or viremia alone. If CMV were a direct cause of rejection, it should have been detected earliest, assuming that infection and rejection were able to be detected with similar sensitivity in all cases; however, no consistent temporal relationship was detected, indeed, rejection was detected first in the majority of patients. These findings, which were confirmed in the prospective part of the present study, do not support a major causal role for CMV in post-transplantation cardiac rejection.

Six of the 27 patients in the prospective study developed rejection but remained CMV-negative by culture; they included 5 who were shown to be D−R− at the time of transplantation; 5 of the 6 were found to undergo

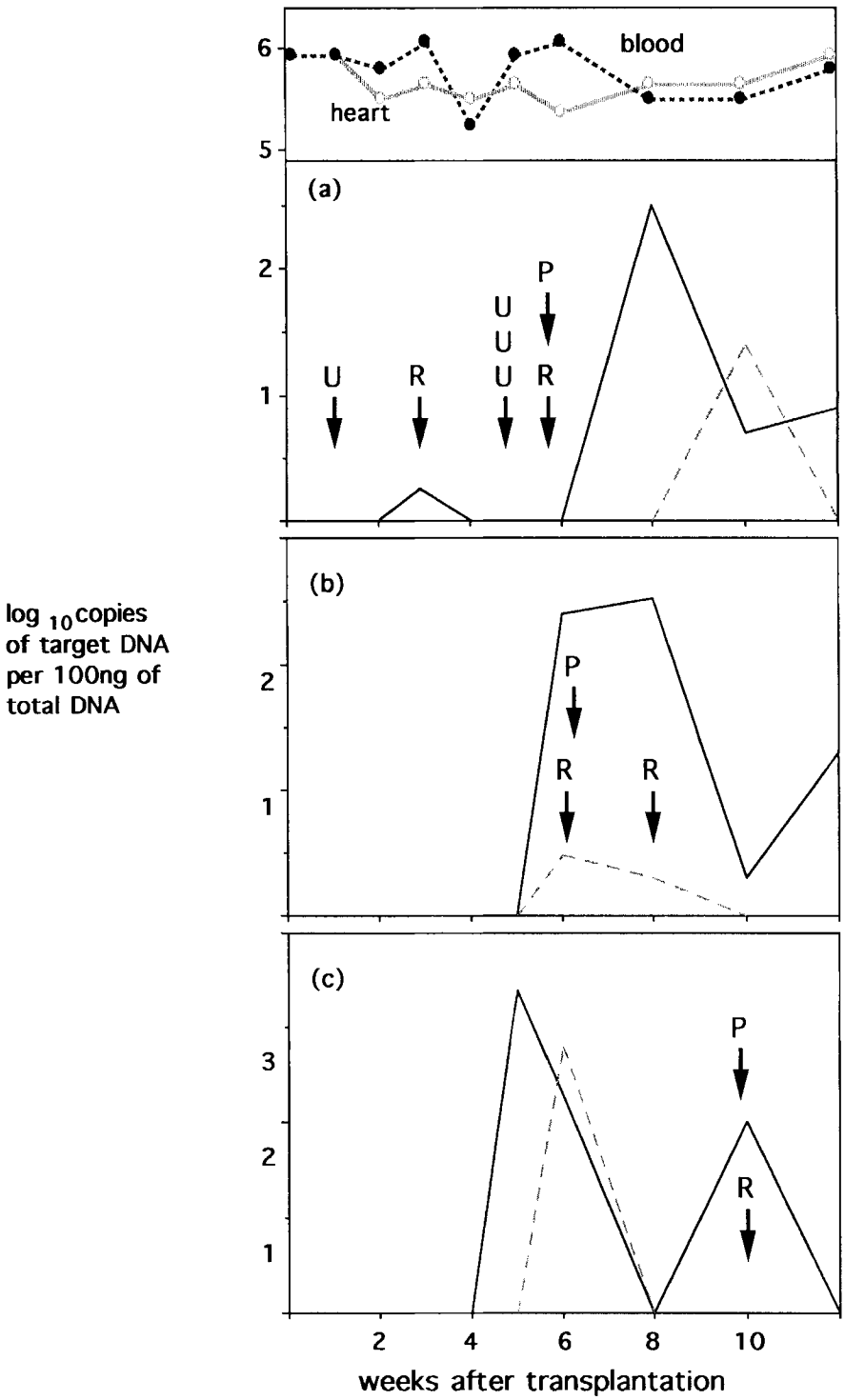

Fig. 4. Development of CMV DNA levels in the heart (solid line) and blood (broken line) of individual patients **(a–c)** with time after transplantation, all 3 patients and their donors being negative for antibody to CMV (D−R−). R = rejection; P = treatment with methylprednisolone; U = unit of blood given. The curves labeled "heart" and "blood" are the results of titrating the samples from patient (a) by single-round PCR for DNA specific for the HRS "housekeeping" gene.

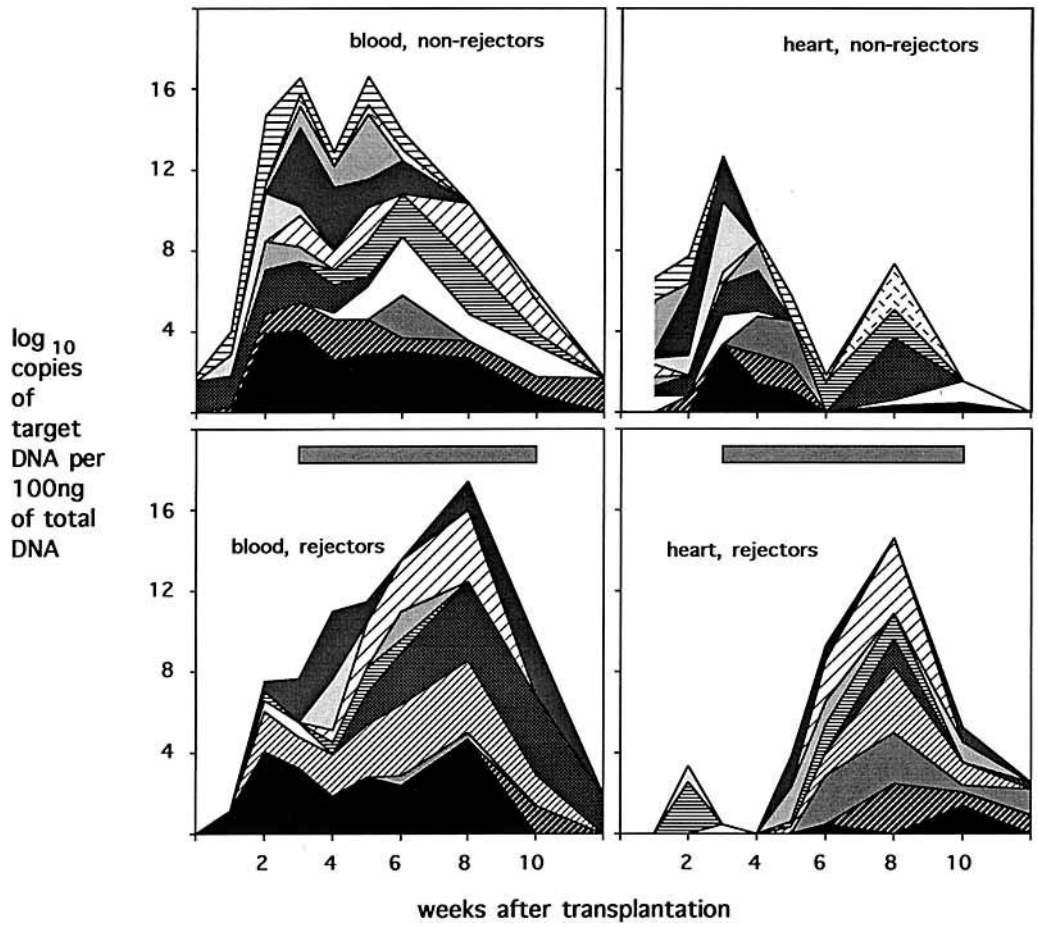

Fig. 5. Levels of CMV DNA in the heart and blood of transplant patients, plotted cumulatively, with time, according to whether or not rejection was detected; the bar above the plots for the samples from the rejectors indicates the time span during which rejection was detected. Non-rejectors: 15 patients; rejectors: 12 patients.

active CMV infection when tested by the *mtrII* nested PCR. Our previous experience has been that the *mtrII* PCR procedure is more sensitive than virus culture for detecting CMV in peripheral blood [Fernando et al., 1994; Rawal et al., 1994]. There were also 6 patients, culture-negative, who did not develop rejection, but of whom 4 underwent CMV infection as shown by the PCR. Studies on murine CMV have shown that tissue samples from infected mice may contain up to 10^6 copies of viral DNA as detected by PCR, yet remain negative by culture [Pollock and Virgin, 1995; Reddehase et al., 1994]. There was no difference between the numbers of CMV DNA-positive patients who were positive or negative for rejection, again indicating no association between CMV infection and rejection.

In a previous retrospective study from this laboratory in which stored biopsy specimens showing different grades of rejection were tested for CMV DNA by a qualitative PCR, significantly more biopsies with moderate-severe rejection than with mild or no rejection were positive for CMV DNA; even so, the degree of association was low (34–37%; [Fernando et al., 1994]) and the finding was not confirmed in the present study. In the previous study also, significantly more biopsies showing moderate-severe rejection than no rejection were positive for CMV DNA; however, this was only in the case of the D+R+ patients, no such difference being detected in the D+R− and D−R+ patients. This, too, was not confirmed in the present study, such inconsistency arguing against a strong association between CMV and rejection. A previous finding, which was confirmed, was that significantly more biopsies were positive for CMV DNA from D−R− patients who developed rejection than from those who did not develop rejection but, on its own, this does not suggest a significant association between rejection and CMV.

Previous longitudinal investigation, by us, of a small number of heart transplant patients, using stored serial biopsy samples, demonstrated no consistent pattern, over time, between the development of rejection and the

detection of CMV DNA [Fernando et al., 1994]; this was also the case in the present prospective study. Moreover, the detection of peaks of CMV DNA at different times after transplantation, in heart and blood, affirmed that CMV DNA levels in the heart were unrelated to levels in the blood. This was not surprising given that most of the total DNA extracted from biopsies must be derived from solid tissue rather than blood, and will include endothelial cells and myocytes, both of which are reported to harbour sequences for CMV [Arbustini et al., 1992]. Attempts to relate the different patterns of appearance of CMV DNA in heart and blood with the development of rejection were unrewarding, but such differences serve to confirm that the transplanted heart itself is a major source of CMV infection, as has been suggested by reports of the detection of CMV sequences in the coronary arteries [Gulizia et al., 1995; Wu et al., 1992; Yamashiroya et al., 1988].

An interesting feature of the PCR was its ability to demonstrate the development of peaks of CMV DNA in patients who were serologically D−R− at the time of transplantation and who thereafter showed no detectable CMV IgG antibody and remained negative for CMV by culture. Similar results were reported in our previous retrospective study [Fernando et al., 1994] and in the prospective study by Lowry et al. [1994]. An obvious explanation could be contamination with extraneous viral DNA during the performance of the PCR; however, careful precautions were taken to avoid this and the peaks of CMV DNA in heart and blood both tended to develop at the same time rather than randomly. Moreover, PCR for CMV MIE and late (gH) gene sequences, although of low sensitivity in our hands, gave positive results on samples containing particularly high levels of CMV *mtrII* DNA. Thus, had contamination been the explanation for the positive *mtrII* PCR results, it would have had to have been with viral genomic DNA rather than *mtrII* PCR product DNA and to have happened when the DNA was being extracted. Increasingly, there are reports of CMV DNA and mRNA being detected by the PCR in healthy CMV antibody-negative subjects including blood donors [Bevan et al., 1991; Ehrnst et al., 1995; Stanier et al., 1989; Taylor-Wiedeman et al., 1991; Zhang et al., 1995] but equally there are other reports which disagree with these findings [Bitsch et al., 1992; Gerna et al., 1991; Jiwa et al., 1989; Urushibara et al., 1995]. All of the patients in the present study had received blood transfusions either during or after the operation, and in the case of the D−R− patients these were always screened for absence of antibodies of CMV. Whether the development of peaks of CMV DNA in the latter patients was the result of exogenous or endogenous infection, the fact remains that the infections they represented were soon overcome without the development of a detectable specific antibody response, suggesting the action of CMV-specific cytotoxic T cells or non-specific cellular defence mechanisms, including phagocytic cells and natural killer cells. Jonjic et al. [1994] have presented evidence that cytotoxic CD4 T cells can restrict the replication and spread of murine CMV in the absence of antibody. Failure to mount a detectable humoral response may reflect the immunomodulatory properties of cyclosporin A [Bretscher and Havele, 1992], which was used routinely in the primary immunosuppressive regimen. An interesting observation was the detection of CMV DNA sequences in the lymph node tissue of a CMV antibody-negative donor, the recipient of whose heart (also CMV antibody-negative) went on to develop CMV infection as judged by the PCR. The possibility of lymph nodes representing a significant reservoir of CMV infection in CMV antibody-positive and -negative individuals deserves further study.

Cumulative analysis of the CMV DNA levels in heart and blood provided an overall impression of the dynamics of the infection in the various categories of patients. Thus, the levels of viral DNA were higher in the blood than the heart; moreover, the cumulative levels in the blood relative to those in the heart were different for D+R+, D−R+, and D−R− patients, confirming that CMV DNA levels in heart and blood were independent of one another. CMV DNA levels in both sites appeared to be bimodally distributed, suggesting the possibility of two successive waves of CMV infection in infected patients. Biphasic manifestation of viral DNA has been reported in mice infected with murine CMV [Collins et al., 1994] and two peaks of specific lymphoproliferative activity have been noted in healthy human volunteers after live CMV vaccine, although within a shorter time interval than here [Plotkin et al., 1989]. The two peaks in the present study suggest the dissemination of CMV from a primary focus of infection, followed by a second wave of viremia, or else an altered trafficking of mononuclear cells which are harbouring CMV.

The cumulative analysis of the PCR results also revealed that in patients who did not experience rejection, peak levels of CMV DNA were attained within the first 6 weeks after transplantation. On the other hand, in those who did develop rejection, CMV DNA appeared later. Many of those who developed rejection showed an increase in CMV DNA as a result of treatment with steroids; those who had rejection, but were not treated with steroids, developed mainly low levels of CMV DNA. The absence of rejection in the patients who developed CMV DNA early on supports the view that CMV infection is not a direct cause of rejection. Rather, the results suggest that CMV DNA levels and rejection are separate indications of the overall immune status of the patient. Thus, patients in whom CMV DNA develops early may be more effectively immunosuppressed than those in whom it develops late. Greater immunosuppression from the outset would favour an early escape of CMV from immune surveillance, would blunt any inflammatory response, and thus forestall severe rejection. Lesser immunosuppression, by allowing a stronger immune response from the start, might both promote rejection and effectively restrain the spread of CMV and, in so doing, prolong the interval between transplantation and the detection of increased levels of CMV DNA. Treatment with steroids, when necessary to reduce rejection, would not

only diminish inflammation but, by so doing, would also upregulate the level of CMV infection by increasing the range and number of host cells permissive for CMV [Lathey and Spector, 1991]. This explanation of our findings suggests, therefore, that CMV infection and rejection are unconnected except insofar as both are modulated by the immune response; indeed, there were several cases of CMV infection without rejection and one case of rejection in the absence of CMV infection. Thus, monitoring of CMV DNA levels may be useful as a guide to the status of the patient's immune response, allowing adjustment of the level of immunosuppression in order to reduce the likelihood of rejection but without provoking CMV infection to the extent of giving rise to clinically significant disease.

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